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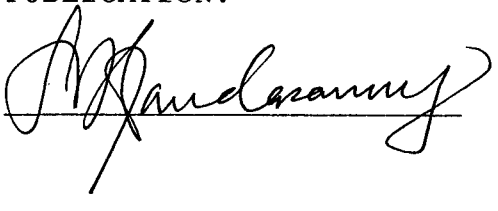

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13. ABSTRACT ***(Maximum 200 Words)***

This is the second annual report of our research project entitled "Methyl-Deficient Diets and Risks of Breast Cancer Among African-American Women: A Case-Control Study by Methylation Status of the ER gene". During the period, the numbers of interviewed cases and controls increased to 243 and 232, respectively. The number of collected tumor tissue specimens reached 177 and more specimens are being obtained. Collected tissue specimens were measured on estrogen receptor status. Most data collected have been entered and maintained timely. We also wrote and submitted a manuscript related to breast cancer research in African-American women. However, laboratory measurement of methylation status of the ER gene CpG islands has lagged based on a number of reasons, despite our endeavors. Generally, this project has progressed according to the statement of work. More efforts will be made on the measurement of methylation status.

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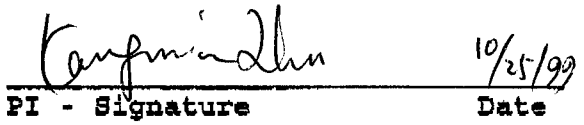

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INTRODUCTION

This is the second annual report of our research project entitled "Methyl-Deficient Diets and Risks of Breast Cancer Among African-American Women: A Case-Control Study by Methylation Status of the ER gene". This project tests our hypothesis that methyl-deficient diets are more likely to be related to breast cancer with methylated ER gene CpG islands.

This study uses a case-control design. Cases consist of African-American female breast cancer patients aged 20-64 and living in Davidson, Shelby and Hamilton counties, Tennessee. Controls are comprised of African-American women without breast cancer who are selected through random-digit telephone dialing and frequency matched to cases by 5-year age range. We interview study subjects for information on dietary methyl-components and other risk factors. We also collect tissue specimens to measure ER levels and the methylation status of the ER genes. The completion of interviews with study subjects, tissue collection, and laboratory measurements is important to the success of the project.

BODY

According to the Statement of Work, the following should have been conducted during the past year: (1) recruitment and interview of eligible patients (cases), (2) recruitment and interview of controls, (3) tumor tissue collection and processing, (4) estrogen receptor measurement, (5) measurement of ER gene methylation, and (6) data entry and management. Except these tasks, we wrote and submitted a manuscript. Our article on the hypothesis of

this study was also published during the period.

1. Recruitment of eligible patients (cases)

The recruitment of cases involves the following steps: (1) identifying eligible breast cancer patients and their doctors through the Tennessee Cancer Reporting System (TCRS), (2) seeking doctors' consents by sending them letters and making reminder calls, and (3) obtaining patients' consent for participating in the study.

1.1. Doctors' consent

After we identify eligible patients and their doctors through TCRS, we mail doctors a letter to get their permission to contact their patients. For doctors who did not respond to the mail, we send the second one. If a doctor did not respond to both mailings, a reminder call is made to contact him/her.

Up to October 10, 1999, TCRS provided us with 764 eligible patients with breast cancer. Out of the patients, 151 had no doctors identified and 33 died. Three hundred and seventy-two doctors were identified for the remaining 580 patients and were contacted. Table 1 summarizes doctors' responses to our letters and remind calls. Because we are still under way to wait for responses from some doctors, these are not final numbers. Based on the statistics in the table, 328 of doctors (88%) responded to our study. Out of the doctors who responded to the study, 249 gave us consent to contact their patients. These doctors provided

us with 396 patients for us to contact.

1.2. Case recruitment and interviews

We mail a packet to patients with a doctor's consent. This packet includes a cover letter introducing the study and a consent form for patients to sign. If a patient did not respond to the packet, we send the second one. A reminder call (where a telephone is available) is made to women who did not reply to both mailings. Table 2 shows the outcomes of our first and second mailings and reminder calls as of October 10, 1999. Again, these are not final numbers because the contacts with some patients are still going on. Among patients to whom we contacted, 164 (42%) agreed and 14 (4%) refused to participate in the study. The rest of them either did not respond to the study or could not be located.

We make home visits to women who did not respond to the mailings and do not have a telephone number available to get their consent. Home visits are conducted by an African-American research team member, nurse, breast cancer survivor, social worker, or local woman with good communication skills. As of October 10, 1999, we visited 161 patients. Eighty percent of women whom we could talk to agreed to participate (table 3). However, a substantial number of women have moved or died.

After mailings, reminder calls, and home visits, 244 patients have agreed to participate as of October 10, 1999. Two hundred and forty-three cases have been interviewed and therefore included in the case group (73 were interviewed in the past year).

2. Control recruitment and interviews

Controls are selected through random-digit dialing (RDD), in which we randomly select one of the telephone prefixes of the cases and add four random-selected digits to constitute a telephone number. Up to 9 calls to this number are made over a two week period (3 day-time, 3 evening, and 3 weekend calls) to find an eligible woman according to ethnic background and age range. For a woman who is eligible and agrees to participate, a telephone interview is conducted. As of October 10, 1999, we have called 15,616 numbers and identified 288 eligible women. Two hundred and thirty-two of these women (81%) have given us consent and have been interviewed (90 were interviewed in the past year).

3. Tumor tissue collection and processing

During the past year, we continued collection of breast cancer tissue specimens from hospitals where cases are pathologically diagnosed. Specifically, we were able to locate the tissue specimens that had been originally identified to be in St. Joseph Hospital, Memphis and had been unable to find due to hospital closing. As of October 20, 1999, we have requested 238 tumor tissue specimens from 17 hospitals in the three counties. Out of 238 specimens requested, we have obtained 181 (76%) (77 were collected in the past year). Out of the 181 specimens, ten were requested again based on unqualified specimens. More specimens are being obtained.

4. Estrogen receptor measurement

ER analysis is carried out using the immunohistochemical method (Chaudhuri et al, 1993, Ferno et al, 1996). Currently, we have stained 168 tumor tissue slides and obtained ER measurement results for 158 of them (10 of the 168 slides contained no tumor tissues or were unsatisfactory, and therefore specimens are requested again). ER staining and reading is being continued.

5. Measurement of ER gene methylation

In our last annual report, we described the MS-PCR method that we would use for the measurement of methylation status of the ER gene CpG islands. This is a highly sensitive and complicated method. Although our laboratory made unremitting efforts in formulating and verifying the procedures using breast cancer cell lines with known ER status, we were unable to obtain ideal and reliable results. A PhD level researcher repeated the procedures a number of times after our research assistant responsible for the laboratory tests left, and still failed to get satisfactory results. Therefore, we decided to seek collaboration from outside researchers who have expertise in the measurement. At the American Association for Cancer Research meeting in April, Dr. Zhu contacted several researchers who have been successful in using the technique for the possibility of collaboration. Dr. Shridhar from Mayo Clinic, who was presenting a study on DNA methylation, would help us in the measurement. After a period of subsequent communications, preparations and DNA extraction from collected breast cancer tissues, her laboratory did some tests using 20 specimens from our study. Due to insufficient

labor and time for Dr. Shridhar's laboratory, we only chose primer pairs 1and 4 for the measurement of methylation status, as opposed to four primer pairs suggested in the last report. Results on primer pairs 1 and 4 should be sufficient for this epidemiological study based on the correlation between the four primer pair sets.

Dr. Shridhar's laboratory used the MS-PCR method with some modifications in DNA modification. For quality control, they used placental DNA methylated with SS1 methylase as the positive control and pure water as the negative control. To show that the DNA modification was successful, they also measured HIC1 gene, a candidate tumor suppressor gene that is frequently hypermethylated and silenced in solid tumors (1,2). Figure 1 shows the results on the positive control, negative control, and HIC1 genes from the specimens, using HIC1 primers. The positive bands can be seen for the positive control and most specimens, but not the negative control, suggesting that the measurement was accurate. Figure 2 shows the results based on methylated primer 1 of the ER gene. Only specimen # 20 had methylated ER gene CpG islands detected. No methylation was found for all specimens when using methylated primer 4 (results not shown).

Measurement based on unmethylated primers has not been conducted, because the technician who was responsible for the tests left for a new position. This has made it difficult to evaluate the current results.

6. Other data collection and quality control activities

We continued obtaining from the Tennessee Cancer Reporting System (TCRS) diagnostic information for all cases. We also sent self-administered oral contraceptive forms to study women. Currently, 253 (53%) out of 475 women completed and returned the form. We also continued our quality-control interviews by randomly selecting women who had been interviewed.

7. Data entry and management

Our databases are well maintained. These include administrative data, tissue collection and measurement data, RDD data, questionnaire data, OC data, and data from TCRS. Most collected data have been entered.

8. Publication and manuscript

We have been in the process of data collection since the beginning of the project. While data were not available for data analysis for this study, we meditated other important research issues on breast cancer in African-American women, the population for this project. As a result, we wrote and submitted a manuscript entitled "African-American ethnicity in epidemiological studies of calcium antagonists in relation to cancer" (attachment 6). In addition, our accepted article on the hypothesis of this project came out last year (attachment 7).

9. Recommendations in relation to the statement of work

9.1. Methylation measurement: Laboratory measurement of methylation status had to pause because of leaving of the technician at Dr. Shridhar's laboratory. Currently, we are not sure whether and when Dr. Shridhar can continue the work. Therefore, while we wait to hear from her, we are also looking for other potential collaborations on the laboratory measurement because of the timeline for the work.

9.2. Case recruitment: In our last report, we mentioned our difficulties in contacting eligible patients as a result of no doctors identified for some patients and some doctors' refusal to participate in the study. We therefore applied for waive of obtaining physicians' consents prior to contacting patients. However, the Advisory Committee, the Tennessee Cancer Reporting System, did not approve the procedure in its meeting in December 1998, although approved earlier by the IRB of Meharry Medical College. Therefore, we had to continue our original procedures in obtaining doctors' consents. As shown earlier, this would substantially reduce the pool of eligible women whom we could contact and decrease the overall participation rate. Thus, we are also recruiting patients diagnosed in 1998, expecting to interview about 300-310 cases at the end of data collection. This will be slightly less than the number expected in the proposal (n=362). The slightly smaller sample size may not substantially reduce the study power.

KEY RESEARCH ACCOMPLISHMENTS

- Obtained consents from doctors and patients through mailing, calling and home visiting, and interviewed 73 cases in the past year;

- Randomly called more than 6,400 telephone numbers to identify controls and interviewed 90 controls in the past year;
- Collected 77 tumor tissue specimens in the past year;
- Measured ER status of collected tumor tissues;
- Partially measured methylation status of the ER gene of 20 specimens; and
- Wrote and submitted a manuscript.

REPORTABLE OUTCOMES

1. Submitted manuscript entitled "African-American ethnicity in epidemiological studies of calcium antagonists in relation to cancer"

2. Published article, "Methyl-deficient diets, methylated ER genes and breast cancer: an hypothesized association"

CONCLUSIONS

According to the statement of work, we were supposed to continue identifying/recruiting/interviewing study subjects, collecting breast cancer tissue specimens, measuring ER status and methylation status of the ER gene CpG islands, and entering and maintaining data during the period. The research team has made its effort to identify and interview study subjects as many as possible. Despite the difficulties in the recruitment of study subjects due to lack of doctors' consents for a substantial proportion of eligible patients and extra endeavors in obtaining consent from women, we have obtained 244 cases and 232

controls, about two third of expected numbers. The collection of tumor tissue samples has been going well with 76% of requested specimens collected (excluding those for which we are still waiting). ER status has been measured and data have been entered and maintained timely. However, laboratory measurement of methylation status of the ER gene CpG islands has lagged because of technical difficulties and unexpected leaving of the technician from the collaborating institution that took over the measurement. Recruitment of study subjects and laboratory measurement of methylation status will be our working priorities in the next year. We will try our utmost to get these works done well.

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APPENDICES

1. Table 1. Doctors' responses according to the first mailing, second mailing and reminder call
2. Table 2. Patients' responses according to the first mail, second mail and reminder call
3. Table 3. Home visits to women who did not respond to the study and the outcomes
4. Figure 1. MS-PCR analysis of HIC1 using HIC1 primers
5. Figure 2. MS-PCR analysis of the ER CpG island using ER primer 1
6. Attachment 1: Manuscript entitled "African-American ethnicity in epidemiological studies of calcium antagonists in relation to cancer"
7. Attachment 2: Published article, "Methyl-deficient diets, methylated ER genes and breast cancer: an hypothesized association"

Table 1. Doctors' responses according to the first mailing, second mailing and reminder call

	Doctor not responded*	Doctor responded*	Status of patients** with doctor's response		
			Agreed to contact	Refused to contact	Patient died
1st mail	241	131	206	22	20
2nd Mail	192	49	68	15	3
Reminder call(s)	144	148	122	114***	10

*, number of doctors; **, number of patients, *** including patients with doctors who did not want to be involved in the study at all and doctors who did not respond.

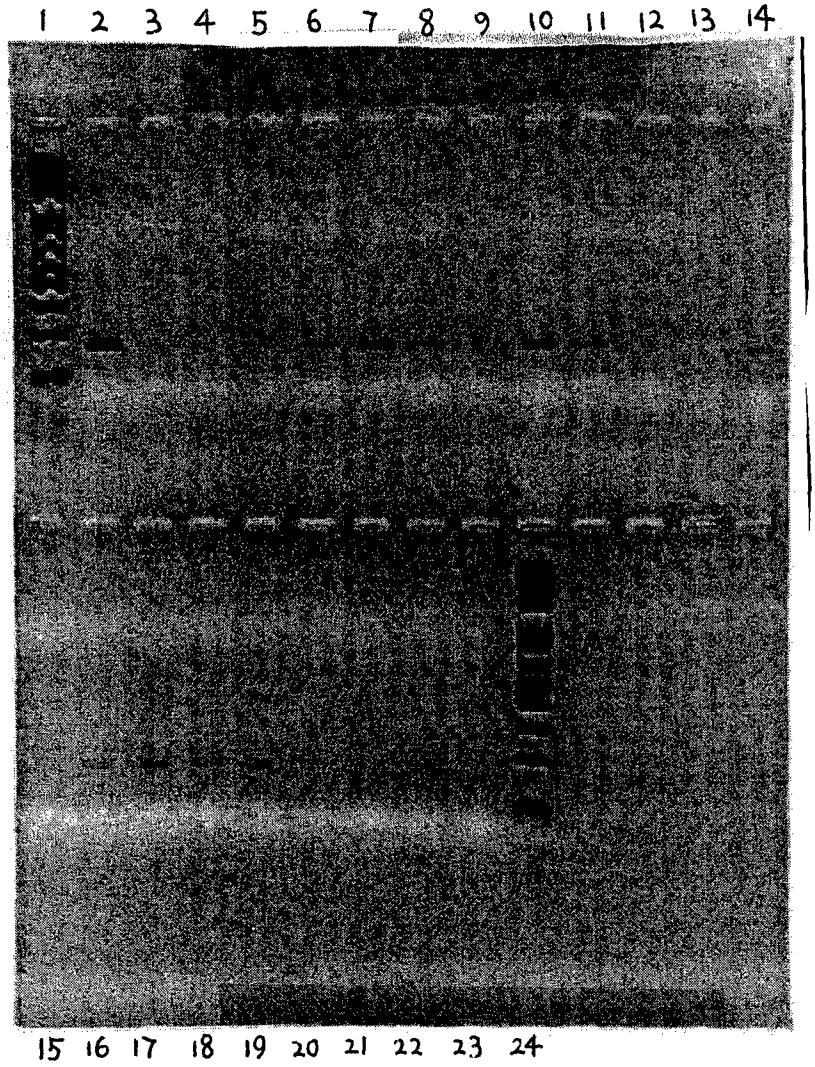
Table 2. Patients' responses according to the first mail, second mail and reminder call

	Women responded		Women not responded		Total
	Agreed to participate	Refused to participate	Unable to locate	Other	
1st mail	60	1	9	326	396
2nd Mail	57	0	0	269	326
Reminder call	47	13	11	46	117*

*, the number of patients with a telephone number available.

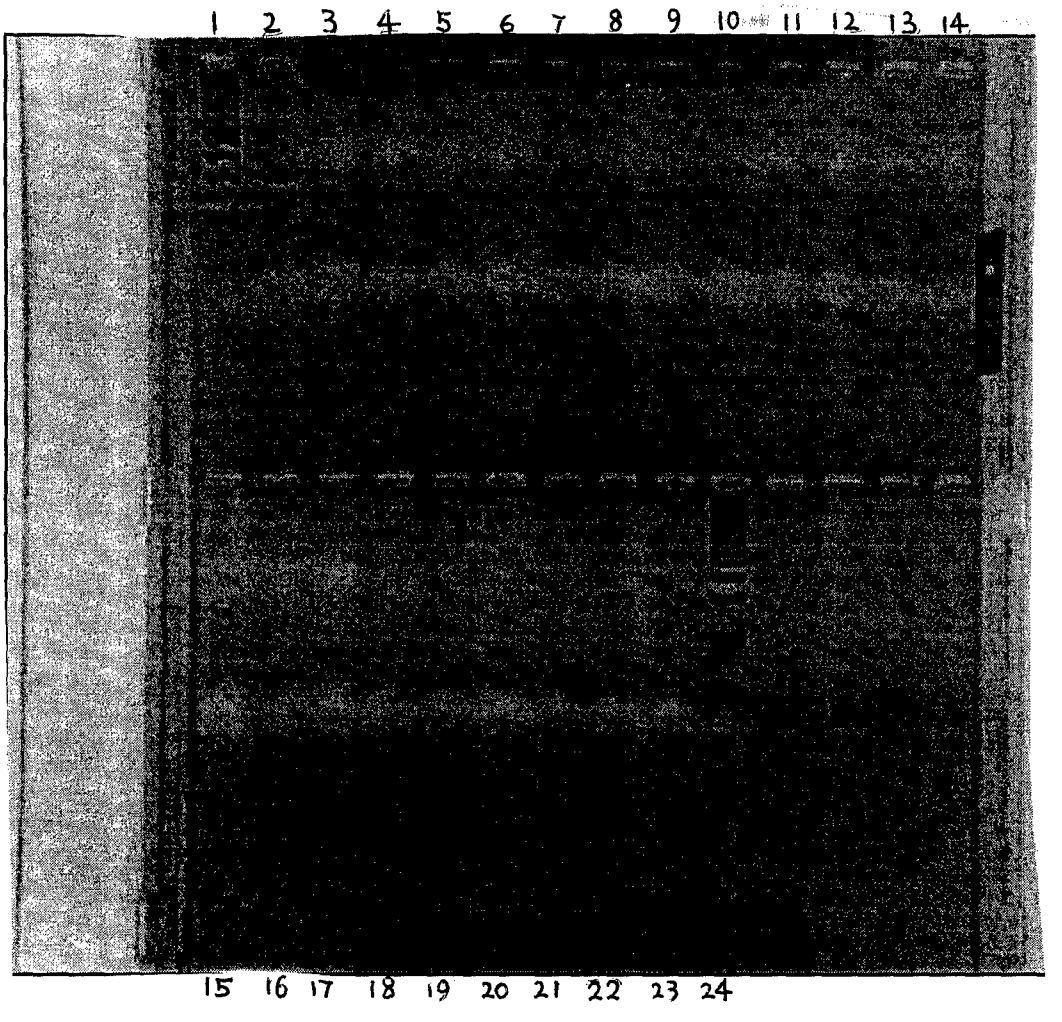
Table 3. Home visits to women who did not respond to the study and the outcomes

# Homes visited	
# Women whom we were able to talk to	101
# consents	81
# Refusals	18
# Poor health status	2
# Women whom we were unable to talk to	60
# Died	14
# Moved	28
# Unknowns	8
# Miscellaneous	10



Column 1: Ladder
Column 2: Placenta (positive control)
Columns 3-22: Specimens 1-20
Column 23: Water (negative control)
Column 24: Ladder

Figure 1. MS-PCR analysis of HIC1 using HIC1 primers



Column 1: Ladder
Column 2: Placenta (positive control)
Columns 3-22: Specimens 1-20
Column 23: Water (negative control)
Column 24: Ladder

Figure 2. MS-PCR analysis of the ER CpG island using ER primer 1

African-American Ethnicity in Epidemiological Studies of Calcium Antagonists in Relation to Cancer

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UNPUBLISHED MANUSCRIPT THAT SHOULD BE PROTECTED

Calcium antagonists (CAs) are a class of drugs that are primarily used for treatment of hypertension and ischemic heart diseases. Since a substantial proportion of the elderly either use or have used CAs because of high frequencies of the cardiovascular diseases, any adverse effects might have considerable impact on the health status of the population. Whether the use of calcium antagonists heightens the risk of cancer has been an increasing concern in recent years. Up to now, there have been a number of epidemiological studies on the relationship between CAs and cancer.[1-9] While there have been inconsistent results, most of the studies have assessed the relationship in Caucasian populations.

Only one study has examined the hypothesized association between CAs and cancer among not only Caucasian but also African-American people.[1] In this cohort study, people who were or were not taking CAs were followed up to four years and compared in the risk of cancer. While Caucasian patients taking CAs were 1.6 times (95% CI 1.2-2.2) more likely to have developed a cancer than their Caucasian counterparts who did not, the corresponding relative risk estimate was 3.4 (95% CI 1.3-9.4) among African-Americans. This study, while it had a limitation of a small sample size of African Americans, implies that the CA-cancer association may be stronger among African Americans. The postulated higher risk in African-American women is possible because this ethnic population is more likely to respond to CAs and other risk factors and their modifications on the effects of CAs may differ between this ethnic group and other populations. Because of the possibility and a more widespread use of CAs in African Americans, it is desirable to target this population in research on CAs in relation to cancer.

African Americans are more likely to respond to CAs due to possible difference in drug metabolism

Because of differences in genetic, environmental and cultural factors, different racial groups may differ in the ways they respond to and metabolize drugs.[10] For example, interethnic differences in genetically determined polymorphism and drug-metabolizing enzymes have been increasingly recognized.[11-12] It is also shown that dietary or environmental factors may account for some ethnic differences in response to medications.[13] Due to these variances, African Americans may respond to antihypertensive therapy differently from Caucasians.

Studies have suggested that African Americans are relatively resistant to the antihypertensive effects of beta-blockers or angiotensin converting enzyme (ACE) inhibitors but respond well to calcium antagonists.[14-15] Compared with beta-blockers or ACE inhibitors, blood pressure levels are decreased significantly more with CA treatment in African Americans,[16] while Caucasians respond better to beta-blockers and ACE inhibitors.[17] It has also been shown that the percentage of hypertensive patients who respond to CA is higher among African Americans than Caucasians. In a meta-analysis of 48 studies on the antihypertensive efficacy of calcium antagonists, Messeri et al. found that as the proportion of African-American hypertensive patients increases, the mean reduction in blood pressure increases,[18] implying that African Americans more likely respond to CAs.

Recent studies suggest that better response to CAs in African Americans may result from accelerated cellular calcium turnover (enhanced calcium entry into and accelerated calcium

extrusion from the cytosol) in the population.[19] Fekete et al. demonstrated that Ca2+ fluxes (entry and extrusion) were greater in lymphocytes from African Americans than in those from Caucasians.[20] The increased cellular calcium turnover rate may result in the amplified sensitivity to CAs. The other factor that may account for the different responses is cytochrome P450 (CYP) enzyme system that is important for the drug metabolism. The main cytochrome P450 enzymes that can mediate the metabolism of CAs include CYP3A4, CYP1A2 and CYP2C.[21-24] Although African Americans and whites have not been compared on CYP3A4 and CYP2C, CYP1A2 oxidative activity has been found to be lower in African Americans than in Caucasians.[25] Studies on other CYP enzymes have shown that CYP1A1 gene inducibility is significantly lower in African Americans[26] and there are African-American-specific polymorphism in the CYP genes such as CYP2D6 [27] and CYP1A1[28] that may give rise to changed or no activity towards drug substrates. These studies suggest that, although more evidence is needed on CYP3A4 and CYP2C, the CYP system is less effective in African-Americans than in whites.[29] Therefore, the effects of CAs may be longer or stronger in African Americans due to reduced P450 activities.

While African Americans have greater response to the therapeutic effects of CAs, they may also be more vulnerable to non-therapeutic effects of the medications. If CAs increase the risk of breast cancer, the increase may be greater for African-American women because they may be more prone to the effects of CAs.

Different ethnical groups may differ in other factors and possible interactions between the factors and CAs

Response to medicines is affected by not only differences in drug metabolism but also by differences in susceptibility to drug effects (such as a specific side effect) which is determined by genetic as well as non-genetic factors. It is well known that there are black/white differences in genetic traits and non-genetic factors. In genetic factors, for example, Caucasians always have a positive reaction and African Americans have a negative reaction to the Duffy antigens that are encoded by the Duffy gene.[30] African-Americans and Caucasians may also differ quantitatively in some genetic characteristics such as the prevalence of certain alleles of specific genes.[31] The differences in genetic materials may contribute to differential susceptibility to disease (e.g. a drug side effect) between the two ethnic groups.[31] They may have different risk for cancer from certain non-genetic exposures (such as drug use) as a result of variegation in genetic predisposition.

Because of socio-cultural differences, African Americans and Caucasians may also be exposed to different non-genetic factors that may influence the risk of cancer and the metabolism of CAs. For instance, environmental hazards, dietary intake and obesity are inequitably distributed between African Americans and Caucasians.[32-34] These factors have been allegedly associated with increased risk of some cancers.[35-38] Non-genetic factors may also influence the response to medicines. As shown in studies in animals and humans, diets may change the bioavailability and action of CAs.[39-41] Obesity may increase volume of distribution of CA [42] and decrease the activity of CYP3A4 [43] that metabolizes CAs. As a result of potential effects of non-genetic factors on the risk of cancer and metabolism of CAs, the association of CAs with cancer may vary depending upon the existence or level of the factors.

The relation of genetic and non-genetic factors on their effects on cancer is complex. Furthermore, either or both of the factors may interact with CAs. The complexity of the interrelationship between CAs, genetic factors and other non-genetic factors may make the association of CAs with cancer different between different ethnic groups. African Americans are more likely to have adverse exposures (such as environmental hazards, unhealthy diets and obesity) and more likely to be susceptible to the effects of CAs. Because many of risk factors act synergistically with each other, it is possible that an association between CAs and cancer may only exist or is stronger in African Americans.

African Americans are more likely to use CAs and studies targeting this population are warranted

It is well known that African Americans have a higher prevalence of hypertension than white Americans.[44-45] A follow-up report from the National Health and Nutrition Examination Survey found that the incidence of hypertension among African Americans was at least twice that among Caucasians for nearly every age- and sex- matched group.[44] African Americans also have more severe form of hypertension and worse consequences of hypertensive disease.[45] They are more likely to have left ventricular dysfunction, stroke, and renal damage,[44] which need medications that can lower vascular peripheral resistance, promote sodium excretion, and improves renal hemodynamics. CAs may meet these requirements [44] and therefore may be especially good for African-American patients.

In 1993, the National Committee on Detection, Evaluation, and Treatment of High Blood Pressure suggested that calcium antagonists may often be preferred for treatment of hypertension in African Americans.[46] Physicians who treat African Americans may have followed these guidelines. Therefore, CAs may be used more frequently among African Americans due to a higher prevalence of hypertension or more recommendations by doctors.

Espeland et al. [47] conducted a cross-sectional survey among hypertensive persons aged 60-80 who subsequently entered a clinical trial evaluating the safety and effectiveness of noonpharmacologic therapy for the treatment of mild and moderate hypertension. This study was conducted in Baltimore , Maryland, Memphis, Tennessee, New Brunswick, New Jersey, and Winston-Salem, North Carolina, from September, 1992 through July, 1994. They found that 38.5% of African-Americans were using CAs. The corresponding percentage was 34.2% for other races (>98.5% were Caucasian). After controlling for differences in other variables related to the CA use, African-Americans were 1.27 times more likely to take CAs than Caucasians (95% CI 1.04-1.54).

Fritzpatrick et al. [5] studied two cohorts aged 65 and older: the original female cohort (most of study subjects were white) enrolled during 1989-1990 and African-American female cohort enrolled during 1992-1993. About ten percent of women from the original cohort took CAs at enrollment. For the African-American cohort, the proportion of women who took CAs at recruitment was 28.2%. These two percentages can not be compared directly because the use of CAs has increased over time. However, the data do suggest that the frequency of CA use may be high in general elderly African-American populations.

Owing to widespread use of and greater response to CAs, and possible interactions of the medication with other factors in African Americans, studies in this population are needed. Inter-individual variability in drug response is well known: the same dose may induce toxicity for some people while it even does not have therapeutic effect for others. It can be conceived that the variability is more obvious between different ethnic groups due to larger genetic and non-genetic differences. An epidemiological study recruiting different ethnic populations would show discrepant magnitudes of relative risk due to different response to the medication or discrepant population attributable risks due to different prevalence of CA use. Because factors may interact in different ways for various cancers, the modifications on the effects of CAs of factors related to interethnic differences may vary for different cancers. Thus, different cancers should be defined in future studies. Because the use of CAs is modifiable and because African-American women are more likely to use CAs and have higher mortality rate of cancer, whether this medication increases the risk of cancer in the population is an important public health issue.

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Methyl-deficient diets, methylated ER genes and breast cancer: An hypothesized association

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Recent molecular studies show that ER-negative breast cancer results from the lack of ER gene transcription due to the methylation of the CpG island 5′ to the gene. Because CpG island methylation is an early event in carcinogenesis and because methyl-deficient diets could result in CpG island methylation, it is relevant to postulate that methyl-deficient diets may be a risk factor for breast cancer with methylated ER genes (as opposed to the disease with unmethylated ER genes). This molecular-based etiologic hypothesis may facilitate epidemiological research on the relationship between breast cancer and diet that has been unclear until now. *Cancer Causes and Control,* 1998, 9, 615-620

Key words: Breast cancer, estrogen receptors, gene, methyl-deficient diets, methylation.

Introduction

Breast cancer can be divided into two types according to the tumor estrogen receptor (ER) level: ER-positive or ER-negative. Because the presence or absence of ERs in breast cells may differentially affect the role some risk factors, such as estrogens, play on the etiology of the disease, it is reasonable to hypothesize that risk factor profiles of breast cancer vary by ER status of the disease. However, previous epidemiological studies on risk factors by ER status have obtained inconsistent results.[1-8] Recent molecular studies show that ER-negative breast cancer results from the lack of ER gene transcription due to the methylation of the CpG island 5′ to the gene.[9,10] We suggest that this observation may be critical in assessing breast cancer risk factors according to the ER status of the tumor.

The inconsistency in previous epidemiological studies by ER status may be related to problems in using total ER levels as an indicator of ER status without fully understanding the basis of ER level variation. Moreover, it is possible to misclassify an individual's ER status by just measuring total ER levels. For example, a tumor with a sparse distribution of ER-positive cells may be falsely considered ER-negative and a tumor with a dense distribution of ER-negative cells may be falsely considered ER-positive. This patchiness or variegation and failure to understand the underlying cause of ER level variation may have affected study results and conclusions.

Using the methylation status of the ER genes is less likely to be prone to the same effects of cellularity in defining ER status, and may help define a molecular-based etiologic hypothesis of breast cancer. Because CpG island methylation is an early event in carcinogenesis and may relate to breast cancer's lack of ER

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expression and because diets deficient in methyl-groups (such as methionine choline, and folate) can result in abnormal DNA methylation/carcinogenesis, it is reasonable to postulate that methyl-deficient diets may be a risk factor for breast cancer with methylated ER genes, but not for the disease with unmethylated ER genes.

CpG island methylation is an early event in carcinogenesis

CpG islands are located in the promoter regions of genes and their methylation status is important in gene transcription.[11-13] Active transcription requires an unmethylated state of 5′ sites that exist in normal adult tissues.[12,14,15] When CpG islands are methylated, chromatin structure can change, causing genes in these chromosome regions to become transcriptionally inactive.[16] These chromosome alterations may also result in DNA instability leading to tumorogenesis. In a study on colon cancer, Makos *et al.*[16] found that there is abnormal methylation of the CpG island areas on 17p in colon adenomas and the abnormality increases in colon cancers. Because allelic losses of chromosome 17p are characteristics of colon carcinomas, the results suggest that methylation precedes these allelic losses. Another study of colorectal tumors[14] showed that CpG island methylation of the ER gene increases with age in human colonic mucosa from normal individuals and can be found in all colorectal tumors. Vertino *et al.*[15] examined whether the aberrant methylation of CpG islands evolves as a function of immortalization and oncogene-induced neoplastic transformation of bronchial epithelial cells. They found that the methylation of CpG islands at 17p13 occurred during the immortalization of normal human bronchial cells and preceded oncogene-induced transformation. Because chromosome 17p13 deletions occur in lung tumorigenesis,[17] the results suggest that the methylation appears early in bronchial epithelial cell carcinogenesis that is related to immortalization.[15] In addition, Vertino *et al.*[13] found that aberrant CpG island methylation appeared during normal aging of fibroblasts and may predispose some cells to transform into cancer. Combined, these studies imply that CpG island methylation is an early event of carcinogenesis.

CpG island methylation of the ER gene may cause low ER expression in breast cancer

The human ER gene is located on chromosome 6q24-q27.[18,19] Recent studies have shown that ER-negative breast cancer is caused by a lack of ER gene transcription.[18,20] The lack of ER gene expression is related to methylation of the 5′ region of the gene:[21] 4 out of 5 samples were hypermethylated in ER-negative carcinomas and 13 of 15 were hypomethylated in ER-positive carcinomas.[20] Using human breast cancer cell lines, it was subsequently demonstrated that methylation of the CpG island in the 5′ region and first exon of the gene is responsible for lack of expression of ER gene in ER-negative breast tumors.[9] This was confirmed by reactivating the ER gene using inhibitors of DNA methylation, which demethylate the ER CpG island.[10] Although the results based on breast cancer specimens are more complex due to the heterogeneity of cell populations within a tumor, it was found recently that ER-negative tumors have higher mean scores of ER CpG island methylation than ER-positive tumors.[22] By analogy to the colorectal cancer story given above, ER gene methylation may be an early event in some breast cancer (*i.e.* ER negative), if breast cancer shares similar molecular mechanisms to other tumors.

Risk factors may differ depending upon the methylation status of the ER genes

Certain risk factors may be important for tumors with methylated genes and other factors may be significant for other tumors. For example, in a recent study,[23] it was found that lung cancers from smokers and from animals exposed to tobacco-specific carcinogens had a low incidence of CpG island methylation of the ER genes, while lung cancers of non-smokers and spontaneous tumors in animals had a high incidence of methylation. For breast cancer, it can be postulated that factors that can cause or facilitate CpG island methylation of the ER gene may only increase the risk of breast cancer with ER gene methylation. Due to a lack of receptors resulting from the methylation, breast cells with methylated CpG islands may not be affected by subsequent exposures to estrogens during their transformation into cancer cells. On the contrary, tumors with unmethylated ER genes, and therefore with receptors, may be more susceptible to factors that can interact with ERs. If these differences exist, breast cancers with and without ER gene methylation will have distinct risk factor profiles.

Methyl-deficient diets could result in abnormal DNA methylation and therefore are more likely to be related to breast cancer where the ER gene CpG islands are methylated

No studies have been conducted on breast cancer risk factors according to the methylation status of the ER gene. However, the possibilities discussed above imply an association of methyl-deficient diets with breast cancers where the ER gene is methylated. Such an

association, if it exists, may be based on the following hypothesized mechanisms. It is suggested that diets deficient in methyl-groups (such as methionine and folate) or high in methyl group antagonists (such as alcohol) cause increased DNA methyltransferase (DNA-MTase) activity.[24] There may be two types of DNA-MTase activities: *de novo* methyltransferase activity and maintenance methyltransferase activity.[25] Elevated *de novo* DNA-MTase activity may initiate[25,26] and elevated maintenance DNA-MTase activity may subsequently spread and maintain[26] methylation of the usually unmethylated CpG sites, possibly through the disruption of the boundaries that normally protect CpG islands from methylation.[26] Methylation of the CpG sites after a relatively long-term methyl-deficient diet has been directly demonstrated during the transition to tumor in animals,[27] although it was not suggested in a study in humans,[28] in which short-term dietary methyl group restriction was used and methylated urine metabolites rather than methylation of the CpG sites was measured. The hypermethylation of the CpG islands silences tumor suppressor genes[29] such as the ER gene[30] and therefore is related to the occurrence of cancer. Methyl-deficient diets can also lower cellular levels of the methyl donor S-adenosylmethionine.[31-33] Reduced S-adenosylmethionine can cause global genomic hypomethylation[25,32,34,35] and therefore the activation of some oncogenes.[26] Decreased S-adenosylmethionine can also facilitate the activity of DNA-MTase as a mutator enzyme, leading to CpG mutagenesis.[25] Probably as a result of these DNA changes, diets reducing methyl-group availability may increase the risk of cancer. Observations in animal models[36] and humans[24,37,38] support this. In Giovannucci *et al.*'s study,[24] a combination of high alcohol and low methionine and folate intake conferred a relative risk of 7.4 for distal colon cancer. Because low dietary methyl-components may cause (1) the methylation of ER gene CpG islands that reduces tumor suppressing activities of the ER genes, (2) global genomic hypomethylation that may activate some oncogenes and (3) CpG mutagenesis, it is reasonable to hypothesize that methyl-deficient diets and those high in methyl-antagonists are likely to be related to breast cancer primarily with methylated ER genes. Figure 1 depicts the hypothesized association.

Our hypothesis of the association between methyl-deficient diets and breast cancer with ER gene methylation suggests the need to study breast cancer risk factors with respect to specific molecular characteristics. Tumors with and without a specific molecular characteristic may have different causal pathways and therefore have different risk-factor profiles. The differences may originate from two things. First, the change in methylation pattern is probably not inherited. Rather, it may result from a number of environmental or somatic factors that do not co-occur in cancers without this molecular change. Second, even though methylation changes exist (due to either environmental exposures or somatic factors), they may not cause cancer alone. It is likely that methylation imparts susceptability to cells and causes cancer in the presence of other genetic or environmental factor(s). Because these other factors have their effects in conjunction with this susceptibility, their association with cancer would be different, depending upon whether a tumor has such susceptibility.

Several issues should be considered in the exploration of the relationship between methyl-deficient diets, breast cancer and methylation status of the ER gene. First, methyl-deficient diets are also associated with global genomic hypomethylation related to the occurrence of cancer. If the hypomethylation could occur without methylation of CpG islands, breast cancer without methylated ER genes may also be susceptible to the effects of methyl-deficient diets. While we do not exclude this possibility, it is unlikely because widespread genomic hypomethylation and methylation of CpG islands usually exist simultaneously in tumor cells.[26] Second, the metabolism of methyl groups is influenced by methylenetetrahydrofolate reductase (MTHFR).[39,40] A mutation in the MTHFR gene, which is common in many populations,[41] can reduce specific MTHFR activity, leading to decreased methionine and S-adenosylmethionine levels.[42] Decreased S-adenosylmethionine in individuals with the MTHFR mutation appears only in the presence of low folate status.[43] Therefore, the association between methyl-deficient diets or methyl-antagonists and cancer might be stronger among people with mutated MTHFR genes, as suggested by recent studies.[44,45] The effect of methyl-deficient diets on breast cancer with methylated ER genes, if any, may be modified by the MTHFR genotype, which should be considered in studies on the hypothesized association. Finally, the hypothesized association between methyl-deficient diets and the risk of breast cancer with methylated ER gene is based on the hypothesis that breast cancers with and without methylated ER genes are two different entities that may have different etiologic pathways. This hypothesis is tenable because the methylation status of CpG islands has been suggested as an early event in the development of cancer. However, if methylated CpG islands also occur as a function of tumor progression after a tumor develops, they may appear in some late-stage breast cancers that were unmethylated at their early stage, leading to the misclassification of real methylation status. Early-stage tumors should be used if this is true.

Many epidemiologic studies of cancer risk factors have not distinguished tumors by genetic or epigenetic

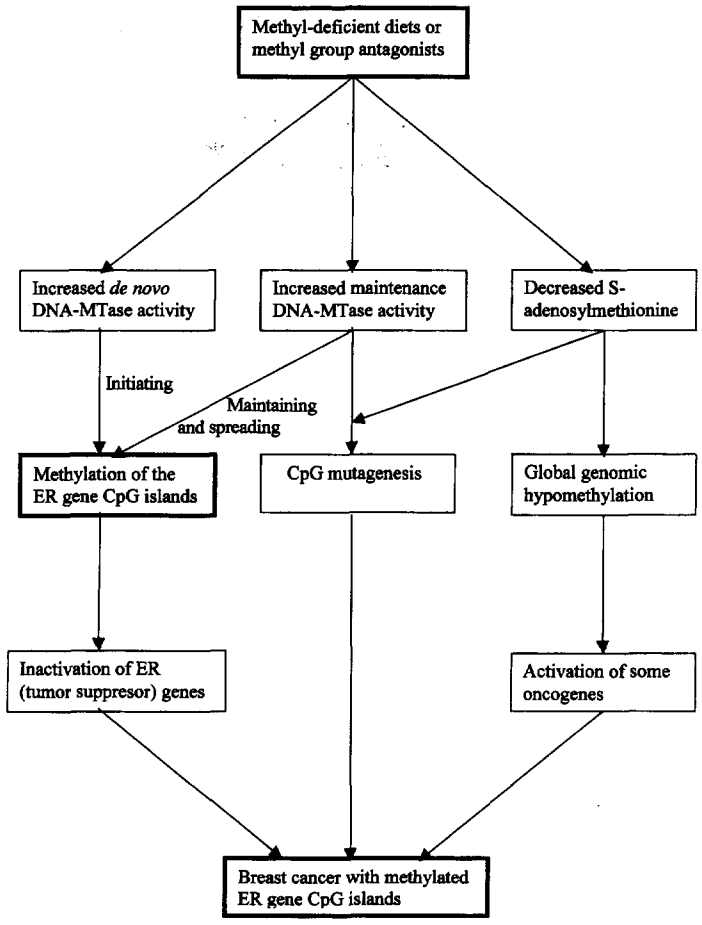

Figure 1. Hypothesized association between methyl deficient diets and breast cancer with methylated ER gene CpG islands.

characteristics.[46] The pooling of similar cancers having different causal pathways would dilute the ability to detect risk factors for each pathway. Without information on the methylation status of ER gene CpG islands, previous epidemiological studies on fruits/vegetables (rich in folate[24,37]) and poultry/fish/dairy products (rich in methionine[24]) have found either an association between the lack of these dietary factors and increased risk of breast cancer,[47-51] or no association.[52,53] Studies on alcohol consumption (a methyl group antagonist) also have showed a null or weak positive association with breast cancer.[54-56] Because methyl-deficient diets and methyl group antagonists are related to abnormal DNA methylation, they may be a risk factor for tumors with methylated ER genes, but not for those without. The lumping of tumors with different ER gene methylation statuses may have led to an estimate of a diluted association. Case-control studies on methyl-deficient diets, in which breast cancers are distinguished by the methylation status of the ER genes, can be used to explore such a possibility. Cohort studies are also feasible by examining the ER methylation status of tumors among women with and without methyl-deficient diets. Studies that distinguish different genetic or epigenetic status of tumors would improve research on the relationship between risk factors and the disease,[57,58] increasing our ability to comprehend diet-breast cancer relationships that have not been clear to this point.

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REPLY TO
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2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
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